



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12N 15/87, A61K 48/00	A1	(11) International Publication Number: WO 94/06922 (43) International Publication Date: 31 March 1994 (31.03.94)
(21) International Application Number: PCT/US93/09031 (22) International Filing Date: 23 September 1993 (23.09.93) (30) Priority data: 07/950,789 24 September 1992 (24.09.92) US 08/042,870 5 April 1993 (05.04.93) US (71) Applicant: THE UNIVERSITY OF CONNECTICUT [US/US]; Storrs, CT 06269 (US). (72) Inventors: WU, George, Y. ; WU, Catherine, H. ; 52 Rid- gebury Road, Avon, CT 06001 (US). (74) Agents: MANDRAGOURAS, Amy, E. et al.; Lahive & Cockfield, 60 State Street, Boston, MA 02109 (US).		(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: METHOD OF ENHANCING AND/OR PROLONGING EXPRESSION OF A GENE INTRODUCED INTO A CELL		
<div style="text-align: center;"> </div>		
(57) Abstract The expression of polynucleotide introduced into a cell by means of a targeted complex of the polynucleotide linked to a cell-specific binding agent can be enhanced and prolonged by inhibiting translocation or fusion of endosomes to lysosomes.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NE	Niger
BE	Belgium	GN	Guinea	NL	Netherlands
BF	Burkina Faso	GR	Greece	NO	Norway
BG	Bulgaria	HU	Hungary	NZ	New Zealand
BJ	Benin	IE	Ireland	PL	Poland
BR	Brazil	IT	Italy	PT	Portugal
BY	Belarus	JP	Japan	RO	Romania
CA	Canada	KP	Democratic People's Republic of Korea	RU	Russian Federation
CF	Central African Republic	KR	Republic of Korea	SD	Sudan
CG	Congo	KZ	Kazakhstan	SE	Sweden
CH	Switzerland	LJ	Liechtenstein	SI	Slovenia
CJ	Côte d'Ivoire	LK	Sri Lanka	SK	Slovak Republic
CM	Cameroon	LU	Luxembourg	SN	Senegal
CN	China	LV	Latvia	TD	Chad
CS	Czechoslovakia	MC	Monaco	TG	Togo
CZ	Czech Republic	MG	Madagascar	UA	Ukraine
DE	Germany	ML	Mali	US	United States of America
DK	Denmark	MN	Mongolia	UZ	Uzbekistan
ES	Spain			VN	Viet Nam
FI	Finland				

METHOD OF ENHANCING AND/OR PROLONGING EXPRESSION
OF A GENE INTRODUCED INTO A CELL

Background of the Invention

The benefits of gene therapy for alleviating or correcting defects in the production of proteins are widely recognized and a number of gene delivery systems have been developed. One method involves using viruses to deliver the exogenous gene to a cell but this method introduces viral genes along with the new gene and undesirable viral effects may be produced.

Another method involves targeting a polynucleotide to a cell in vivo where it is then expressed. This can be accomplished using a soluble complex of two linked components: 1) a polycation, e.g., poly-L-lysine, that can bind a polynucleotide in a strong but non-damaging interaction and 2) a ligand which can be targeted specifically to a cell surface molecule unique to the cell. See Wu, G.Y. and Wu, C.H. (1988) J. Biol. Chem. 263:14621-14624. The complex specifically binds the targeted cell and is internalized by endocytosis resulting in the incorporation of the polynucleotide.

During endocytosis, the macromolecule to be ingested is enclosed by a small portion of the plasma membrane which pinches off to form an intracellular vesicle or endosome. Newly synthesized lysosomes from the Golgi apparatus are translocated and fused to endosomes. After fusion, the body is called an endolysosome which then develops into a mature lysosome. Lysosomes contain a wide variety of degradative enzymes and, therefore, material in lysosomes can be broken down.

Although transient gene expression may be desirable for some applications of gene therapy, in many other applications, persistent or enhanced expression of the polynucleotide would be required. There exists a need for a method of introducing DNA into cells in vivo so that expression can be enhanced and made to persist. One method for persistent gene expression is to induce replication of the targeted cell(s) (U.S. Patent Application Serial No. 588,013, filed September 25, 1990). Zenke et al. recently reported that chloroquine can be used to raise the pH in the targeted cells' lysosomes during "transfer-rinfection" thus inhibiting degradation of the exogenous gene within lysosomes and increasing the amount of gene incorporated by the cell. See Zenke, M. et al. (May 1990) PNAS USA 87:365-3659. Some investigators have demonstrated that polynucleotide incorporation during transferrinfection can be enhanced by cointernalization of the carrier-poly-nucleotide complexes with certain viruses. See Curiel et al. (October 1991) PNAS USA 88:8850-8854.

Summary of the Invention

This invention pertains to a method of enhancing the amount of a targeted polynucleotide which is internalized by a cell and of prolonging expression thereof. A soluble molecular complex is used to target a polynucleotide to a specific cell in vivo or in vitro. The amount of the polynucleotide internalized is enhanced and/or prolonged by inhibiting translocation or fusion of endosomes to lysosomes.

The molecular complex comprises a polynucleotide complexed to a carrier which is a conjugate of a cell-specific binding agent and a polynucleotide-binding agent. The cell-specific binding agent is specific for a cellular surface structure, typically a receptor, which mediates internalization of bound ligands by endocytosis, such as the asialoglycoprotein receptor of hepatocytes. The cell-specific binding agent can be a natural or synthetic ligand (for example, a protein, polypeptide, glycoprotein, etc.) or it can be an antibody, or an analogue thereof, which specifically binds a cellular surface structure which then mediates internalization of the bound complex. The polynucleotide-binding component of the conjugate is a compound such as a polycation which stably complexes the polynucleotide under extracellular conditions and releases it under intracellular conditions so that the polynucleotide can function within the cell.

Preferably, the complex of the polynucleotide and the carrier is stable and soluble in physiological fluids. It can be administered in vivo where it is selectively taken up by the target cell via the surface-structure-mediated endocytotic pathway.

Translocation or fusion of endosomes to lysosomes can be inhibited by an agent such as a drug or molecule which inhibits microtubule formation. A preferred agent for inhibiting microtubule formation is colchicine. The method of this invention is useful in gene therapy and in other applications where enhanced expression of a polynucleotide introduced into a cell is desired.

Brief Description of the Figures

Figure 1 shows the carrier-DNA complex was infused intravenously 30 minutes after intraperitoneal injection of 0.75 mg/kg colchicine (—●—) or 20 minutes before 66% hepatectomy (—◆—). Control rats received an intraperitoneal injection of the vehicle (dimethylsulfoxide) 30 minutes before DNA administration (—○—). Rats were sacrificed at time points indicated in the abscissa.

Figure 2 shows serum bilirubin levels in rats receiving pSV3-hBUGT₁, a vector for expression of bilirubin-UDP-glucuronosyltransferase: Gunn rats were injected with colchicine (0.75 mg/kg, intraperitoneally) 30 minutes before infusion of the carrier-DNA complex. Three groups of results are shown: (a) Control rats: no treatment (—○—); (b) colchicine only (—◆—); and (c) colchicine followed by DNA targeting (—●—). Blood was collected from tail veins at time points indicated on the abscissa and serum bilirubin level was determined as described in the exemplification.

Detailed Description of the Invention

A soluble, targetable molecular complex is used to selectively deliver a gene to a target cell or tissue. An agent is administered in conjunction with the molecular complex to inhibit translocation or fusion of endosomes to lysosomes and decrease degradation of the molecular complex.

The molecular complex comprises the gene to be delivered, complexed to a carrier made up of a binding agent specific for the target cell and a gene-binding agent. The complex is selectively taken up by the target cell and the polynucleotide is incorporated by the cell.

The gene can be DNA, RNA or an analogue thereof. Typically, it comprises a structural gene encoding a desired protein in a form suitable for processing by the target cell. For example, the gene encodes appropriate signal sequences which provide for trafficking to intracellular destinations or cellular secretion of the product. The signal sequence may be the natural sequence of the protein or exogenous sequences. The structural gene is linked to appropriate genetic regulatory elements required for expression of the gene product by the target cell. These include a promoter and, optionally, an enhancer element operable in the target cell. The gene can be contained in an expression vector such as a plasmid or a transposable genetic element along with the genetic regulatory elements necessary for expression of the gene and secretion of the gene-encoded product.

The carrier component of the complex is a conjugate of a cell-specific binding agent and a polynucleotide-binding agent. The cell-specific binding agent specifically binds a cellular surface structure which mediates its internalization into cellular endosomes by, for example, the process of endocytosis. The surface structure can be a protein, polypeptide, carbohydrate, lipid or combination thereof. It is typically a surface receptor which mediates endocytosis of a ligand. Thus, the binding agent can be a natural or synthetic ligand which binds the receptor. The ligand can be a protein, polypeptide, glycoprotein, glycopeptide or glycolipid which has functional groups that are exposed sufficiently to be recognized by the cell surface structure. It can also be a component of a biological organism such as a virus, cells (e.g., mammalian, bacterial, protozoan) or artificial carriers such as liposomes.

The binding agent can also be an antibody, or an analogue of an antibody such as a single chain antibody, which binds the cell surface structure.

Ligands useful in forming the carrier will vary according to the particular cell to be targeted. For targeting hepatocytes, glycoproteins having exposed terminal carbohydrate groups such as asialoglycoprotein (galactose-terminal) can be used, although other ligands such as polypeptide hormones may also be employed. Examples of asialoglycoproteins include asialoorosomucoid, asialofetuin and desialylated vesicular stomatitis virus. Such ligands can be formed by chemical (e.g., sulfuric acid) or enzymatic (e.g., neuraminidase) desialylation of glycoproteins

that possess terminal sialic acid and penultimate galactose residues. Alternatively, asialoglycoprotein ligands can be formed by coupling galactose terminal carbohydrates such as lactose or arabinogalactan to non-galactose bearing proteins by reductive lactosamination.

For targeting the molecular complex to other cell surface receptors, other types of ligands can be used, such as mannose for macrophages (lymphoma), mannose 6-phosphate glycoproteins for fibroblasts (fibrosarcoma), intrinsic factor-vitamin B12 and bile acids (See Kramer *et al.* (1992) *J. Biol. Chem.* 267:18598-18604) for enterocytes and insulin for fat cells. Alternatively, the cell-specific binding agent can be a receptor or a receptor-like molecule, such as an antibody which binds a ligand (e.g., antigen) on the cell surface. Such antibodies can be produced by standard procedures.

The polynucleotide-binding agent complexes the gene to be delivered. Complexation with the gene must be sufficiently stable to prevent significant uncoupling of the polynucleotide extracellularly prior to internalization by the target cell. However, the complex is cleavable under appropriate conditions within the cell so that the polynucleotide is released in functional form. A non-covalent bond based on electrostatic attraction between the polynucleotide-binding agent and the gene provides extracellular stability and is releasable under intracellular conditions.

Preferred polynucleotide-binding agents are polycations. These positively charged materials can bind noncovalently with the negatively charged polynucleotide to form a soluble, targetable molecular

complex which is stable extracellularly but releasable intracellularly. Suitable polycations are polylysine, polyarginine, polyornithine, basic proteins such as histones, avidin, protamines and the like. A preferred polycation is polylysine (e.g., ranging from 3,800 to 60,000 daltons). Other noncovalent bonds that can be used to releasably link the polynucleotide include hydrogen bonding, hydrophobic bonding, electrostatic bonding alone or in combination such as, anti-polynucleotide antibodies bound to polynucleotide, and strepavidin or avidin binding polynucleotide containing biotinylated nucleotides.

The carrier can be formed by chemically linking the cell-specific binding agent and the polynucleotide-binding agent. The linkage is typically covalent. A preferred linkage is a peptide bond. This can be formed with a water soluble carbodiimide as described by Jung, G. et al. (1981) Biochem. Biophys. Res. Commun. 101:599-606. Alternative linkages are disulfide bond, thioether linkage, or strong noncovalent linkages as in avidin-biotin coupling.

The linkage reaction can be optimized for the particular cell-specific binding agent and polynucleotide-binding agent used to form the carrier. Reaction conditions can be designed to maximize linkage formation but to minimize the formation of aggregates of the carrier components. The optimal ratio of cell-specific binding agent to polynucleotide-binding agent can be determined empirically. When polycations are used, the molar ratio of the components will vary with the size of the polycation and the size of the polynucleotide-binding

agent. In general, this ratio ranges from about 10:1 to 1:1. Uncoupled components and aggregates can be separated from the carrier by molecular sieve or ion exchange chromatography (e.g., Aquapore™ cation exchange, Rainin).

In one embodiment, asialoorosomucoid-polylysine conjugate is formed with the crosslinking agent 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide. After dialysis, the conjugate is separated from unconjugated components by preparative acid-urea polyacrylamide gel electrophoresis (pH 4-5). The conjugate can be further purified on the carboxymethyl functionalized column. See U.S. Patent Application Serial No. 08/043,008 entitled "Method for Preparing Conjugates of Receptor-Specific Ligands and Polycationic Polypeptides", filed April 5, 1993

, the teachings of which are incorporated by reference herein.

The gene can be complexed to the carrier by a stepwise dialysis procedure. In one embodiment, for use with carriers made of polycations such as polylysine, the dialysis procedure begins with a 2M NaCl dialyzate and ends with a 0.15M solution. The gradually decreasing NaCl concentrations results in binding of the polynucleotide to the carrier. In some instances, particularly when concentrations of the polynucleotide and carrier are low, dialysis may not be necessary; the polynucleotide and carrier are simply mixed and incubated.

The molecular complex can contain more than one copy of the same polynucleotide or one or more different polynucleotides. The molar ratio of polynucleotide to the carrier conjugate can range from about 1:100 to about 1:200, depending upon the type and size of carrier and polynucleotide. The ratio can be determined for the particular carrier and polynucleotide by the gel retardation assay described in U.S. Patent No. 5,166,320.

For in vitro use cultured cells can be incubated with the molecular complex of this invention in an appropriate medium. It can also be used similarly ex vivo to enhance transfection of cells which have been removed from an organism and will subsequently be returned to the organism.

For in vivo use, the molecular complex of this invention can be administered parenterally. Preferably, it is injected intravenously. The complex is administered in solution in a physiologically acceptable vehicle such as sterile saline.

The target cell is treated with an agent in sufficient amount to inhibit translocation or fusion of endosomes to lysosomes. The agent can be a drug or molecule which blocks the formation of cellular microtubules. Such a drug or molecule can accomplish this by inhibiting the polymerization of tubulin molecules to form microtubules (e.g., colchicine) or by taking up much of the free tubulin in the cell to form microtubules and stabilizing them (e.g., taxol). Other agents include vinblastine, vincristine, and vindesine. For in vivo gene therapy, the agent is preferably administered intravenously prior to the intravenous administration of the complex.

Timing of the administering of the agent in vivo is important to prevent inhibition of the internalization process which is also dependent on function of microtubules. The agent should be administered before the complex, preferably about 30 minutes to one hour before.

The method of this invention can be used in gene therapy to selectively deliver a gene to a target cell in vivo for enhanced and/or prolonged expression. For example, a normal gene can be targeted to a specific cell to correct or alleviate a metabolic or genetic abnormality caused by an inherited or acquired defect in a corresponding endogenous gene. The method can also be used to enhance or prolong expression of genes encoding therapeutic proteins or genes encoding antisense constructs directed against expression of an endogenous or exogenous gene. The enhancement of the foreign gene expression makes practical the periodic, but not too frequent, administration of a gene.

This invention is illustrated further by the following examples.

EXAMPLES

Example 1

An asialoglycoprotein-polycation conjugate consisting of asialoorosomucoid coupled to poly-L-lysine, was used to form a soluble DNA complex capable of specifically targeting hepatocytes via asialoglycoprotein receptors present on these cells. The DNA comprised a plasmid containing the gene for hepatitis B virus surface antigen. Colchicine was used to enhance and prolong gene expression in the target cell.

Expression Vector Containing Gene Encoding Hepatitis B Virus Surface Antigen

Plasmid pSVHBVsurf was obtained from Dr. T. Jake Liang (Massachusetts General Hospital, Boston, MA; Liang, T.J. *et al.* (1993) J. Clin. Invest. 91:1241-1246). The plasmid (approximately 3.6 kbp) is a pUC derivative containing the SV40 origin of replication and the open reading frame for hepatitis B surface antigen (as part of a 1984 bp insert) driven by the SV40 promoter. The plasmid was cloned in E. coli and purified as described previously (Birnboim, H.C., and Doly, J. (1979) Nucleic Acids Res. 7:1513-1518). Purity was checked by electrophoresis through agarose gels stained with ethidium bromide (Maniatis, T., Fritsch, E.F., and Sambrook, G. *in* Molecular Cloning, A Laboratory Manual, Fritsch, E.G. and Maniatis, T., eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY pp 150-161 (1982)).

Asialoorosomucoid

OR was prepared from pooled human serum (Wu, G.Y. and Wu, C.H. (1988) J. Biol. Chem. 263:14621-14624; Whitehead, D.H. and Sammons, H.G. (1966) Biochim. Biophys. Acta 124:209-211) and dissolved in water. An equal volume of 0.2 NH_2SO_4 was added to the OR solution and the resulting mixture heated at 80°C for 1 hour in a water bath to hydrolyse sialic acids from the protein. The acidolysis mixture was removed from water bath, neutralized with NaOH, dialyzed against water for 2 days and then lyophilized. The thiobarbituric acid assay of Warren was then used to verify desialylation of the OR (Warren, L. (1959) J. Biol. Chem. 234:1971-1975). Also see generally Warren, L. (1959) J. Clin. Invest. 38:755-761 and Schmid, K. *et al.* (1967) Biochem. J. 104:361-368.

The Targetable DNA Carrier

ASOR was coupled to poly-L-lysine (Sigma Chemical Co., St. Louis, MO) Mr = 41,100 (1:1 molar ratio) at pH 7.4 using 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (Pierce Chemical Co., Rockford, IL). The conjugates were purified by cation exchange chromatography using a high pressure liquid chromatographic system (Rainin) employing an Aquapore C-300 column (Rainin) and stepwise elution with 1 M urea, 0.1 M sodium acetate pH 5.0, 2.5, 2.25, and 2.0. The second peak eluted from the column as detected by U.V. absorption at 230 nm was determined as the optimal conjugate (Jung, G. et al. (1981) Biochem. Biophys. Res. Commun. 101:599-606).

The optimal proportion of DNA to mix with the conjugate to form a soluble complex was determined using gel retardation assay as described previously (Wu, G.Y. and Wu. C.H. (1987) J. Biol. Chem. 262:4429-4432). Briefly, samples containing equal amounts of DNA in 0.15 M NaCl were mixed with increasing amounts of the conjugate in 0.15 M NaCl to determine the conjugate to DNA molar ratio which completely retards DNA migration in the gel after filtration through 0.45 micron membranes. The amount of conjugate needed to bind 50-75% of the DNA was calculated and used to form the molecular complex in order to ensure solubility of the complex. To form the soluble molecular complex, the conjugate solution was added very slowly to the DNA by a peristaltic pump at a speed of 0.1 ml/min with constant mixing. An aliquot was taken and absorbance at 260 nm was determined to monitor the amount of DNA. Another aliquot was taken and run on an agarose gel to verify

the formation of complex. The solution containing the complex was filtered through a 0.45 μ membrane filter. Aliquots were taken for testing by agarose gel electrophoresis as described above.

Timing of Colchicine Administration

Groups of 150 g female rats (Sprague-Dawley), two each, were anesthetized with ketamine-xylazine. Groups of rats were injected intraperitoneally with colchicine in sterile phosphate buffered saline, 0.75 mg/kg, one hour prior to, at the time of, and one hour after intravenous (slowly via the tail vein) injection of the complex which contained 2 mg of DNA. A fourth group of rats received only the complex. After 24 hours, the rats were bled and serum was obtained for assay of hepatitis-B virus surface antigen (HBsAg). (Auszyme Monoclonal, EIA Kit for detection of HBV - Abbott). The U.V. absorbance of the resultant solution color change was measured at 492 nm for 200 μ l of serum.

Results

In control rats, the mean of HBsAg detected by ELISA on the first day after injection was 0.42 ± 0.72 ng/ml. Treatment with colchicine, one hour prior to, at the time of, or one hour after injection of the DNA complex resulted in mean HBsAg levels of 2.06 ± 3.55 ng/ml, 1.26 ± 1.53 ng/ml and 0.30 ± 0.30 ng/ml, respectively. These data show that treatment with colchicine one hour prior to injection of DNA complex produced the highest levels of gene expression at 24 hours.

To determine the time course of expression of the targeted gene, 4 control rats without colchicine were used for a day 1 time point and 2 for a day 16 time point. For a time course of the effects of colchicine on the duration of expression, 8 rats were used for a day 1 time point and 3 each for day 4 and day 20 points. The results revealed that rats that received complexed DNA only had surface antigen levels of $0.45 \pm .41$ on day 1, but declined to 0.06 ± 0.00 ng/ml on day 16. In contrast, animals pretreated with colchicine 1 hour prior to injection resulted in levels of 2.29 ± 1.20 on day 1, 0.89 ± 0.12 on day 4 and 0.22 ± 0.13 ng/ml on day 20. These data indicate that colchicine not only increases the level of expression early in the time course but also results in longer and higher levels of expression.

Example 2

An ASOR-PL conjugate was used to target a plasmid containing the gene for bilirubin-UDP-glucuronosyltransferase to hepatocytes of Gunn rats. Colchicine was used to prolong expression.

Animals

Male Sprague-Dawley rats (150-200 g) and bilirubin-UDP-glucuronosyltransferase (B-UGT) deficient Gunn rats (150-225 g) were obtained from a colony at the Albert Einstein College of Medicine. The rats were maintained on standard laboratory rat chow in a 12 hour light/dark cycle.

Colchicine Administration

In experiments to determine the effect of colchicine on hepatic microtubules, colchicine (0.5 mg/ml of dimethylsulfoxide) in doses of 0.25 mg to 1.0 mg per kg was injected intraperitoneally into Sprague-Dawley rats. For gene targeting experiments, 0.75 mg/kg colchicine was used 30 minutes before administration of the carrier-DNA complex (see below).

Plasmids

A plasmid, p(9-12)albCAT, containing the gene for bacterial chloramphenicol acetyltransferase (CAT) driven by an albumin promoter/enhancer was provided by James M. Wilson, University of Pennsylvania, Philadelphia. This plasmid, which was previously shown to express CAT activity after targeted delivery to the liver, in vivo (Wu, G.Y. et al. (1991) J. Biol. Chem. 266:14338-14342) was used for determination of the intracellular fate of endocytosed DNA in this study. For transferring B-UGT activity to Gunn rat livers, a plasmid pSVK3-hBUGT₁ was used. This construct was produced by cloning the full-length coding region of human liver B-UGT₁ (Ritter, J.K. et al. (1991) J. Biol. Chem. 266:1043-1047) cDNA downstream to the SV40 promoter region of the pSVK3 backbone (Pharmacia, Uppsala, Sweden). The insert is followed by an SV40 splice site and an SV40 polyadenylation region.

Synthesis of the ASGP-Polylysine Conjugate and
Formation of the DNA-Carrier Protein Complex

ASOR was prepared as described above. ASOR was covalently linked with polylysine (Sigma, average molecular weight 59000) as previously described (Wu, G.Y. *et al.* (1991) J. Biol. Chem. 266:14338-14342 and Wilson, J.M. *et al.* (1992) J. Biol. Chem. 267:11483-11489). To form targetable carrier-DNA complexes, plasmid DNA, 0.5 mg in 1 ml of 2M NaCl was added to the ASOR-polylysine conjugate (containing 0.15 mg ASOR) in 0.6 ml of 2M NaCl at 25°C. The mixture was placed in a dialysis tubing (1.0 cm flat width) with an exclusion limit of 12000 to 14000 dalton and dialyzed successively at 4°C for 30 minutes against 1000 ml of each of the following concentrations of NaCl: 1.5 M, 1.0 M, 0.5 M, 0.25 M and 0.15 M. After the final dialysis, the complex was filtered through 0.45 μ membranes for injection into rats.

Administration of the DNA-Carrier Complex and Partial
Hepatectomy

Rats were anesthetized with ether and the right external jugular vein was exposed. The soluble complex containing 22 pmol of the DNA in a volume of 0.5 ml, was infused into the right external jugular vein, the vein was ligated and the incision was closed. In one group, 66% hepatectomy was performed through a midline incision (Seglen, P.O. (1976) Methods Cell Biol. 13:29-83) under ether anesthesia, 20 minutes after infusion of the DNA-polylysine complex. In preliminary studies, it was determined that 20 minutes was required for maximum internalization of the injected DNA-carrier complex by

the liver. Rats in both control and partial hepatectomy group were killed at 20 minutes, 1 hour, 4 hours, 24 hours and 7 days after infusion of the DNA-carrier complex, the livers were removed and subjected to DNA analysis.

Assay of CAT Activity

CAT activity in the liver homogenates was assayed in triplicate using ^{14}C -chloramphenicol as a substrate and thin-layer chromatographic analysis as previously described (Gorman, C.M. *et al.* (1982) Mol. Cell Biol. 2:1044-1051). The assay was performed in triplicate.

Assay of B-UGT Activity

B-UGT activity in liver microsomal fractions was determined using UDP- ^{14}C glucuronic acid as substrate (5 mM, 0.2 $\mu\text{Ci}/\mu\text{mol}$) (Jansen, P.L.M. *et al.* (1977) J. Biol. Chem. 252:2710-2716). Blanks contained no bilirubin. Bilirubin and its glucuronides were extracted and separated by thin-layer chromatography (Chowdhury, R. *et al.* (1979) J. Biol. Chem. 254:8336-8339) and detected by autoradiography using authentic bilirubin monoglucuronide and diglucuronide as standard. The silica gel containing the bilirubin monoglucuronide and diglucuronide bands were collected by scraping, the pigments were extracted in methanol and radioactivity was determined after appropriate quench correction.

Analysis of Serum Bilirubin and Pigments Excreted in Bile

Serum bilirubin levels were determined as previously described (Trotman, B.W. *et al.* (1982) Anal. Biochem. 121:175-180). For bile pigment analysis, rats were placed under ether anesthesia and bile ducts were cannulated with PE-10 cannulae. The rats were placed in a restraining cage and allowed to regain consciousness and normal body temperature before collecting bile. Bile was analyzed by high pressure liquid chromatography (Spivak, W. and Carey, M.C. (1985) Biochem. J. 225:787-795).

CAT Activity

In the partial hepatectomy and sham operated groups, no CAT activity was detectable in the liver samples at 20 minutes or 4 hours after administration of the plasmid. At 24 hours, CAT activity was detected at approximately 1.0 microunit/mg protein in both sham operated and 66% hepatectomized rats. Seven days after DNA administration, CAT activity was undetectable in sham operated rats but was detected at approximately 1.0 microunit per mg protein in the 66% hepatectomy group. In long-term experiments, CAT activity persisted for at least 60 days (duration of the experiment) (Figure 1).

In rats that received colchicine, 0.75 mg/kg 30 minutes before the administration of carrier-p(9-12)-albCAT complex, CAT activity persisted in the liver at a level of 1.25 to 1.5 microunits/mg protein for at least 60 days (duration of this study) (Figure 1).

B-UGT Activity

Control Gunn rat livers had no B-UGT activity. Gunn rats that received carrier-pSK3-hBUGT complex, 30 minutes after the injection of colchicine, B-UGT activity was detected in the liver for up to 6 weeks (last time point of determination). The enzyme activity ranged from 3 to 5% of the activity in the normal Wistar rat liver. In Gunn rats that received the carrier-DNA complex, but no colchicine, hepatic B-UGT activity was detectable only 24 hours after administration of the DNA.

Excretion of Bilirubin Glucuronides in Bile

Control Gunn rat bile contained no detectable bilirubin monoglucuronide or diglucuronide. In three rats that received colchicine followed by the carrier-DNA complex, bile pigments were analyzed at day 5, day 14 and day 60 after DNA administration. In all cases, bilirubin monoglucuronide and diglucuronide were detected in bile. Bilirubin glucuronides accounted for 9 to 15% of total pigment excretion in bile.

Serum Bilirubin Levels

In untreated Gunn rats and Gunn rats that received colchicine only, there was no significant decrease in serum bilirubin levels (Figure 2). In rats that received the carrier-pSK3-hBUGT complex, but no colchicine, there was also no significant decrease in serum bilirubin levels. In contrast, when the carrier-DNA complex was administered 30 minutes after the injection of colchicine, 0.75 mg/kg, serum bilirubin concentration declined progressively for at

least 5 weeks to 45% of the pretreatment level. After this period the bilirubin level gradually increased to 70% of the pretreatment level in 60 days. In another rat, the serum bilirubin level decreased to 30% of the pretreatment level in 35 days and remained at that level for at least 60 days (duration of this experiment).

Equivalents

Those skilled in the art will be able to recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.

Claims

1. A method of introducing a polynucleotide into a cell comprising contacting a cell with a polynucleotide complex targeted for specific incorporation into the cell, the polynucleotide complex comprising the polynucleotide linked to a cell-specific binding agent which binds a surface molecule of the cell and is internalized into endosomes of the cell; and an agent that prevents the translocation and/or fusion of endosomes to lysosomes.
2. A method of claim 1, wherein, in the administering step, the polynucleotide is DNA.
3. A method of claim 1, wherein, in the administering step, the polynucleotide comprises a gene.
4. A method of claim 1, wherein, in the administering step, the polynucleotide complex further comprises a polynucleotide-binding agent through which the polynucleotide is linked to the cell-specific binding agent.
5. A method of claim 1, wherein, in the administering step, the polynucleotide-binding component is a polycation.
6. A method of claim 5, wherein, in the administering step, the polycation is polylysine.

7. A method of claim 1, wherein, in the administering step, the cell-specific binding agent binds a surface receptor of the cell which mediates endocytotic uptake of the complex.
8. A method of claim 7, wherein, in the administering step, the cell-specific binding agent is a ligand for the asialoglycoprotein receptor of hepatocytes.
9. A method of claim 8, wherein, in the administering step, the ligand is an asialoglycoprotein.
10. A method of claim 1, wherein, in the administering step, the agent is a drug or molecule which inhibits microtubule formation in the targeted cell.
11. A method of claim 10, wherein, in the administering step, the agent is colchicine.
12. A method of introducing a polynucleotide into a cell of an organism, comprising administering to the organism a polynucleotide complex targeted for specific incorporation into the cell, the polynucleotide complex comprising the polynucleotide linked to a cell-specific binding agent which binds a surface molecule of the cell and is internalized into endosomes of the cell; and an agent that prevents the translocation and/or fusion of endosomes to lysosomes.

13. A method of claim 12, wherein, in the administering step, the polynucleotide is DNA.
14. A method of claim 12, wherein, in the administering step, the polynucleotide comprises a gene.
15. A method of claim 12, wherein, in the administering step, the organism is a human.
16. A method of claim 12, wherein, in the administering step, the polynucleotide complex further comprises a polynucleotide-binding agent through which the polynucleotide is linked to the cell-specific binding agent.
17. A method of claim 12, wherein, in the administering step, the polynucleotide-binding component is a polycation.
18. A method of claim 17, wherein, in the administering step, the polycation is polylysine.
19. A method of claim 12, wherein, in the administering step, the cell-specific binding agent binds a surface receptor of the cell which mediates endocytotic uptake of the complex.
20. A method of claim 19, wherein, in the administering step, the cell-specific binding agent is a ligand for the asialoglycoprotein receptor of hepatocytes.

21. A method of claim 20, wherein, in the administering step, the ligand is an asialoglycoprotein.
22. A method of claim 12, wherein, in the administering step, the agent is a drug or molecule which inhibits microtubule formation in the targeted cell.
23. A method of claim 22, wherein, in the administering step, the agent is colchicine.
24. A method of introducing DNA into hepatocytes of an organism, comprising administering to the organism a DNA complex targeted for specific incorporation into hepatocytes, the complex comprising DNA bound to a conjugate of a ligand for an asialoglycoprotein receptor and a polycation capable of complexing the DNA and releasing it within the hepatocyte; and an agent that prevents the translocation and/or fusion of endosomes to lysosomes.
25. A method of claim 24, wherein, in the administering step, the ligand for the asialoglycoprotein receptor is an asialoglycoprotein.
26. A method of claim 24, wherein, in the administering step, the polycation is polylysine.

27. A method of claim 24, wherein, in the administering step, the agent is a drug or molecule which inhibits microtubule formation.
28. A method of claim 27, wherein, in the administering step, the agent is colchicine.

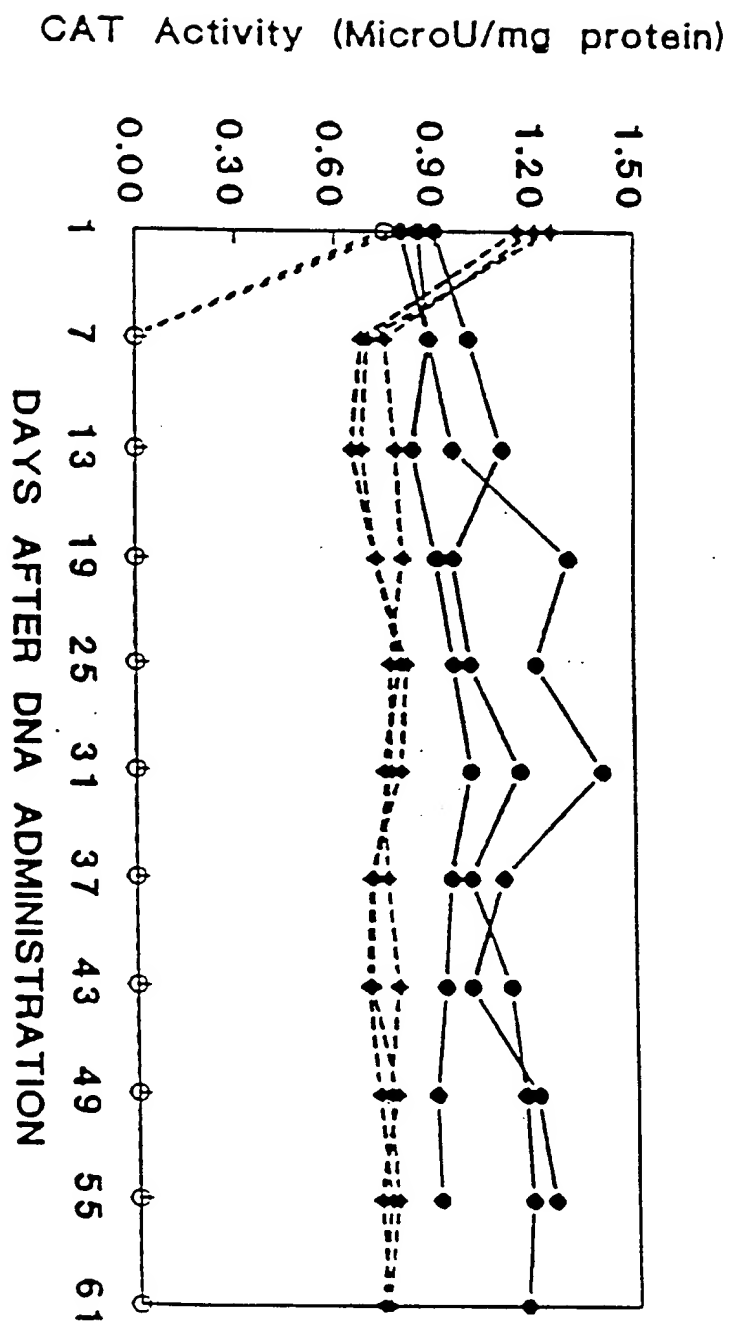


FIGURE 1

SUBSTITUTE SHEET

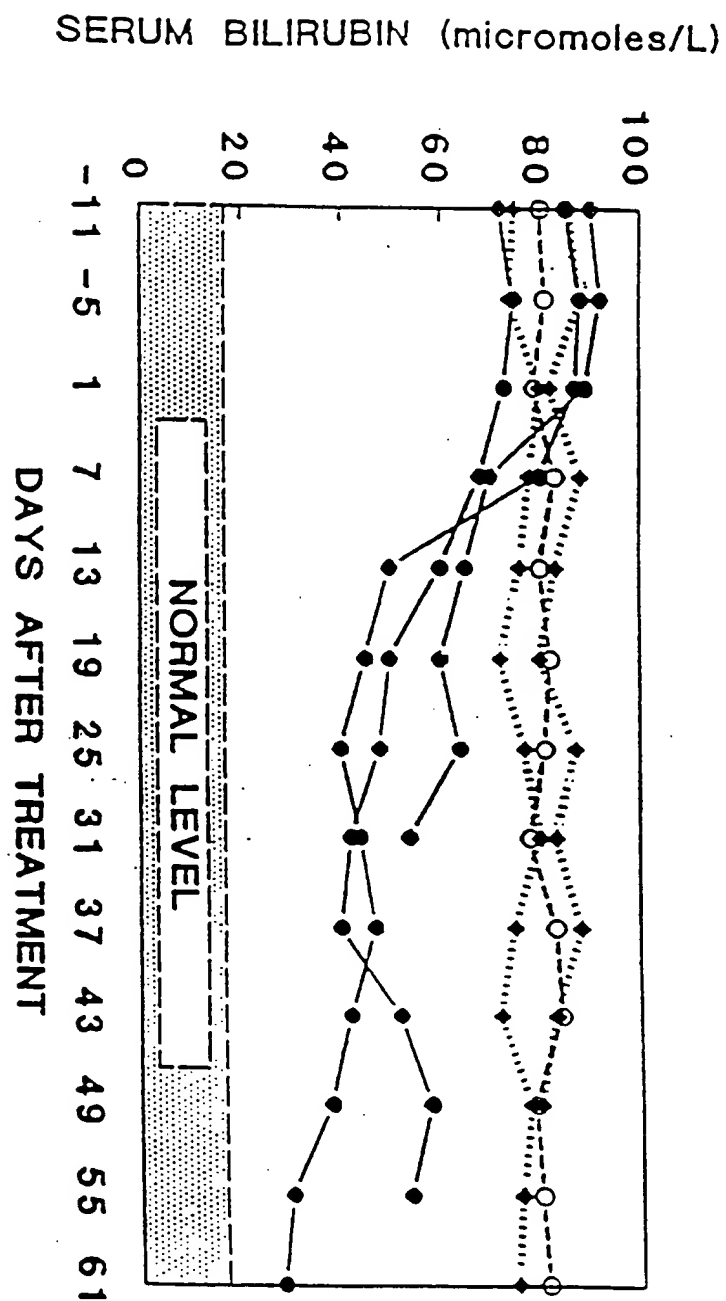


FIGURE 2

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

Int. Application No

PCT/US 93/09031

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/87 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FILE SERVER STN KARLSRUHE, FILE MEDLINE ABSTRACT NO.88195973 & ENDOCRINOLOGY, (1988 MAY), 122 (5), 2044-53 FURLANETTO 'RECEPTOR-MEDIATED ENDOCYTOSIS AND LYSOSOMAL PREPROCESSING OF INSULIN-LIKE GROWTH FACTOR I BY MITOGENICALLY RESPONSIVE CELLS see abstract ---	1-18
Y	STRYER 'BIOCHEMISTRY, THIRD EDITION' 1988, W.H.FREEMAN, NEW YORK, USA, PAGES 944-946 see page 944, paragraph 3 - page 945, paragraph 1 --- -/-	1-18

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

3 December 1993

Date of mailing of the international search report

22 -12- 1993

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Sitch, W

INTERNATIONAL SEARCH REPORT

Int. Patent Application No

PCT/US 93/09031

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,92 05250 (UNIVERSITY OF CONNETICUT) 2 April 1992 cited in the application see whole document, noting especially page 2, lines 2-13, and page 7, line 19-page 8, line 22	1-18
Y	--- PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, USA vol. 87 , 1990 , WASHINGTON, D.C., USA pages 3655 - 3659 ZENKE ET AL 'RECEPTOR-MEDIATED ENDOCYTOSIS OF TRANSFERRIN-POLYCATION CONJUGATES: AN EFFICIENT WAY TO INTRODUCE DNA INTO HEMATOPOIETIC CELLS' cited in the application see page 3655, abstract and paragraph 1 and page 3658, paragraphs 2 and 3 -----	1-18

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 93/09031

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 12-28 (partially) are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.